PATENT
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IN THE UNITED STATES PATENT AND TRADEMARK OFFICE

In re application of:	Confirmation No. 5107
Loeb et al.	Examiner: Lacourciere, Karen A.
Application No.: 09/522,373	Technology Center/Art Unit: 1635
Filed: March 10, 2000	
For: INDUCTION OF VIRAL MUTATION BY INCORPORATION OF MISCODING RIBONUCLEOSIDE ANALOGS INTO VIRAL RNA	DECLARATION UNDER 37 C.F.R. §1.132 OF LAWRENCE A. LOEB
Customer No.: 20350	

Commissioner for Patents
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Sir:

I, Lawrence A. Loeb, M.D., Ph.D., being duly warned that willful false statements and the like are punishable by fine or imprisonment or both (18 U.S.C. § 1001), and may jeopardize the validity of the patent application or any patent issuing thereon, state and declare as follows:

1. All statements herein made of my own knowledge are true, and statements made on information or belief are believed to be true and correct.

2. I have both an M.D. degree from New York University and a Ph.D. degree in biochemistry from the University of California at Berkeley.

3. The invention of the above-referenced patent application provides for the first time a method for increasing the mutation rate of a retrovirus or flavivirus by incorporation of miscoding ribonucleoside analogs. The method comprises the step of administering to a virally infected cell an RNA nucleoside analog, which is incorporated by a polymerase into an RNA copy of a genomic nucleic acid encoding the retrovirus or flavivirus and replaces a naturally occurring nucleotide. The retrovirus or flavivirus is induced to mutate because the RNA nucleoside analog complements a nucleotide that is different from the complement of the naturally occurring nucleotide replaced by the analog.

4. I have read and am familiar with the contents of this patent application. In addition, I have read the Final Office Action, mailed February 13, 2004, received in the present case. It is my understanding that the Examiner believes that the present invention is anticipated by the Larsen *et al.* reference (*Nucleic Acid Research*, 1979, **6**(4):1547-1556), which the Examiner apparently believes describes mutations of viral RNA caused by toyocamycin (TMC), an analog of adenosine.

5. This declaration is provided to demonstrate that the claimed invention of the present application is not anticipated by the Larsen *et al.* reference, because Larsen *et al.* do not describe the mechanism of action recited in the claimed method of the present application.

6. Mutations of nucleic acids occur when nucleotides in one strand of a polynucleotide are altered and their complement nucleotides in the complementary strand are consequently altered through DNA or RNA synthesis. If a nucleotide analog is incorporated into one strand of polynucleotide and replaces a naturally occurring nucleotide, yet the analog's complementary nucleotide remains unchanged in the subsequent synthesis of the complementary strand, then no sustainable mutation has occurred to the nucleic acid. Thus, it is necessary for the operability of the claimed method that a different complement base result from the replacement of a naturally occurring nucleotide by an RNA nucleoside analog.

7. Larsen *et al.* reported evidence of TMC incorporation in viral RNA and speculated that such incorporation might lead to miscoded proteins. However, a later study by the same research group (Hamelin *et al.*, 1982, *Biochemie*, **64**:487-493) indicated that TMC does not affect viral protein synthesis and that there is no evidence of misreading frame resulted from TMC incorporation (top of the left column on page 493 of Hamelin *et al.*). Hamelin *et al.* observed "all the viral messenger RNAs and the viral proteins of a retrovirus synthesized and yet there is no virus release" and thus suggested that TMC might interfere with other cellular functions, such as glycosylation, necessary for viral production (last paragraph in the left column on page 493 of Hamelin *et al.*). This later observation by Hamelin *et al.* strongly indicates to a person skilled in the art that the replacement of adenosines by TMC in viral mRNA does not lead to changes in the incorporation of complementary nucleotides, *i.e.*, does not lead to a sustainable mutation of the nucleic acid.

8. The Examiner appears to take the position that, despite the lack of detectable reading frame shift, Larsen's report of TMC incorporation in viral RNA inherently discloses a mechanism of increasing mutations in viral nucleic acid by causing mis-complementation, *i.e.*, the subject matter of the claimed invention of this patent application. I respectfully disagree with the Examiner.

9. Larsen *et al.* estimated, based on their experimental data, that a significant number of adenosine (rA) in the viral RNA is replaced by TMC (page 1554 of the Larsen reference). If alteration in the complementary nucleotides results from rA → TMC conversion as the Examiner asserts, each viral RNA synthesized subsequent to TMC exposure would contain numerous point mutations. If such point mutations indeed occurred, it is highly probable that they would result in detectable changes to the characteristics of the RNA, such as changes in splicing pattern, reading frame, viral protein synthesis, and/or viral protein activity.

10. However, Hamelin *et al.* reported no detectable changes in viral protein synthesis or reading frame shift as a result of TMC treatment. No change in enzymatic activity was observed when reverse transcriptase from TMC-treated viruses was compared with reverse transcriptase from untreated viruses. See Mauchauffe *et al.*, *Biomedicine*, 1979, **31**:17-20

(attached as Exhibit A), the paragraph bridging pages 18 and 19. In this publication, the authors reported that reverse transcriptase from equal amount of viruses, treated or untreated with TMC, showed no difference in activity when tested using an exogenous oligonucleotide template poly rA-oligo-dT. Although reduced endogenous reverse transcriptase activity was observed in TMC-treated viruses, this reduction was shown to result from alteration of the viral genomic RNA due to TMC incorporation, and not alteration to the reverse transcriptase (the bridging paragraph between pages 18 and 19).

11. Unchanged biological function of viral proteins was also observed in other studies relating to TMC suppression of viral proliferation. For example, Moyer and Holmes (*Virology*, 1979, **98**:99-107, attached as Exhibit B) reported that TMC treatment led to dramatic decrease in viral RNA synthesis vesicular stomatitis virus (VSV); yet all five VSV proteins were synthesized at levels proportional to the amount of mRNA present and appeared biologically functional (see Abstract, pages 104-105, and Table 5 on page 106). In discussing the results of their studies, the authors stated that TMC incorporation is unlikely to affect mRNA translation and further speculated that viral inhibition by TMC may occur by preventing the amplification of progeny RNA synthesis (the paragraph bridging pages 106 and 107). These observations and discussions again lead one of skill in the art to believe that TMC inhibits viral production through a mechanism different from causing point mutations in the viral nucleic acids.

12. Considering the above-mentioned reports together, I find no evidence that TMC incorporation in viral nucleic acids leads to miscomplementation and increased mutations in the nucleic acids. To the contrary, studies indicate that the major effect of rA → TMC substitutions in viral RNA is not that which leads to changes in the sequence of the complementary nucleotides, which are required to cause nucleic acid mutations as defined by the pending claims of this application. The Examiner's assertion that rA → TMC substitutions reported by Larsen *et al.* lead to point mutations in the nucleic acids is not supported by the observations made by several research groups. Without further evidence, one of skill in the art would not believe that such point mutations are a consistent, necessary, and inevitable

occurrence to TMC incorporation in viral nucleic acids and that the accumulation of these point mutations is the mechanism of inhibition of viral RNA synthesis by TMC.

13. In summary, it is my scientific opinion that TMC suppresses the infectivity of a retrovirus, as reported in the Larsen *et al.* reference, via its incorporation into the viral RNA. This incorporation, however, does not appear to lead to the accumulation of mutations of the viral polynucleotide sequence, as defined by the present application. Thus, the action of TMC as described by Larsen *et al.* does not anticipate the claimed invention of the present application, either explicitly or inherently.

Date: 9/10/2004

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Attn: Annette Reese

Biomedicine, 1979, *31*, 17-20.

EFFECTS OF TOYOCAMYCIN ON THE BIOLOGICAL ACTIVITY OF A MURINE ONCORNAVIRUS PRODUCED BY A CHRONICALLY INFECTED CELL LINE

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SUMMARY

Production of Friend virus by chronically infected cells (Friend-Eveline cell line) is depressed by Toyocamycin, an adenosine analogue, at doses which stop cellular growth. The infectivity and the endogenous reverse transcriptase activity of the virions are diminished. These effects are likely to be due to the incorporation of Toyocamycin into the 70S RNA.

RÉSUMÉ

On soumet une lignée cellulaire de souris produisant en permanence le complexe viral de Friend, à l'action de la Toyocamycine, un analogue de l'Adénosine. Dans des conditions où la croissance cellulaire est stoppée, on observe une diminution de la production virale. Les virions produits en présence de l'analogue ont perdu leur pouvoir infectieux et possèdent une activité transcriptase inverse réduite par rapport à celle de virus témoins. Les effets sont très probablement dus à l'incorporation de la Toyocamycine dans le génome viral.

INTRODUCTION

The biology of oncornaviruses is chiefly characterized by integration of their genome via a DNA intermediate into the nuclear DNA of the host cells (1). Viral genes are then expressed according to the same apparent modalities as the cellular genes, since viral m-RNAs possessing properties of cellular m-RNAs such as the capping sequence, and a Poly (A) segment at the 3′ end have been found on polysomes of type C virus-infected cells (4, 6). Among RNA synthesis inhibitors, Toyocamycin (4 amino-5-cyano-7B-D-ribofuranosyl-pyrrolo 2-3-d-pyrimidine) offers a particular interest, as it has been demonstrated to selectively suppress ribosomal RNA synthesis in vitro (11). On the contrary, no apparent effect on m-RNA metabolism was described, since it was found that Toyocamycin did not prevent occurrence of rapidly labeled RNA in cytoplasmic polysomes (12). However, this phenomenon was not studied further (i.e. the functional capacity of this m-RNA was not tested). In this paper, we studied the effect of Toyocamycin (TMC) on the production of virus by a murine cell line chronically infected by the Friend virus. It was found that virions released under TMC treatment were not infectious for reasons which could result from RNA alterations at the level of primary transcription or postranscriptional modifications.

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MATERIALS AND METHODS

Cell cultures and virus purification. The Friend-Eveline cell line (a gracious gift of Pr. Schafer, Tubingen) was grown in suspension in modified Eagle's Medium (MEM) supplemented with 10 % foetal calf serum, 10 % tryptose phosphate and without antibiotics. The supernatants were cleared of cellular debris by a 10,000 g centrifugation for 20 min. Polyethylene glycol 6.000 (Baker) was added to 8 % final (w/v) and the suspension was stirred for 2 h at 4 °C. Pellets were recovered by centrifuging the suspension at 10 000 rpm for 20 min and dissolved in Tris-HCl, 0.01 M, pH 7.4, NaCl 0.1 M, EDTA 0.001 M (NTE) (1/10th to 1/20th of the initial volume). The resulting turbid solution was layered on a 4 ml 20 % glycerol cushion (in NTE buffer) in a Beckman SW-27 rotor tube and centrifuged for 2 hours at 25 000 rpm. Finally, the viral pellet was redissolved in NTE (1 ml) and centrifuged at equilibrium in a 10-70 % sucrose gradient (in NTE buffer) for 17 h at 30 000 rpm (SW-41-Beckman rotor).

Virus titration. The Friend virus produced by the Eveline cell line contains large excesses of helper virus and negligible amounts of spleen focus forming virus (3). Titration of the virus was evaluated by a S+L− test using the FG10 cell-clone isolated by Fishinger et al. (5).

Reverse transcriptase activity. For measuring the endogenous reverse transcriptase activity of the purified virus, the reaction mixtures (100 µl) contained 5 µmoles of Tris (pH : 7.5), 2 µmoles of dithiothreitol, 20 µmoles of KCL, 0.1 µmole of $MnCl_2$, 0.3 µmoles of dATP, dCTP, dGTP, 0.4 nM of [^{3}H] TTP (specific activ. 50 C/mM), 0.02 % of Nonidet P40 (Shell) and 100 µg of purified virus. The mixtures were incubated at 37° and 10 µl aliquots were added at indicated times to 0.5 ml of 0.4 M pyrophosphate and 10 % trichloracetic acid. The precipitates were collected on Millipore filters (0.22 µ) which were dried, immersed in scintillation liquid and counted in a Packard 3390 Counter. Tests using Oligo rA-dT as an exogenous template were performed as previously reported (10).

RESULTS

It has been previously shown that growth of cells in culture is completely stopped by Toyocamycin (11, 12). Since alterations of cellular functions vary with cell origin, we looked for the concentration of antibiotic which blocked growth of the Friend-Eveline virus producing cell line. This was done by adding increasing concentration of Toyocamycin to suspension cultures and determining the number of cells present, 24 and 48 hours after the addition of the drug.

Viability was determined by Blue Trypan dyeing of nuclei. It was observed that cells were arrested in their growth by 0.2 μg/ml Toyocamycin. The cell mortability did not change during the first 24 hours of culture, then progressively increased. At lower concentrations of TMC (0.1 μg/ml), cell growth was not immediately blocked. Higher concentrations provoked efficient cytostatic effect but were too toxic as judged by the cellular destruction which occured during the 24 hours. Moreover, the 0.2 μg/ml concentration inhibited almost totally the biosynthetic pathway of 28S and 18S ribosomal RNA from the nucleus to the cytoplasm (not shown). Accordingly, all the experiments presented in this work were performed using this drug concentration (0.2 μg/ml).

To evaluate the action of Toyocamycin on the virus production, two spinners were seeded with the same number of cells. Toyocamycin (0.2 μg/ml) was added immediately to one, while the other was kept as a control. Twenty-four hours later, the cells were counted to confirm the effect of Toyocamycin, and the supernatants were harvested and processed for virus purification. Five ml of each supernatants were saved for testing biological activity. The PEG pellets were resuspended in NTE buffer and layered on 10-70 % sucrose density gradients prepared in the same buffer and then centrifuged to equilibrium. The results indicated that Toyocamycin-treated cells although stopped in their growth, continued to release virions which banded at the same density as the controls. However, the yield of the virus was lowered two or three fold as compared to the control. This result was still more pronounced when the dose of Toyocamycin was increased and is in accordance with those previously reported (2).

The capacity of TMC viruses to induce foci in monolayers of FG 10 cells was tested. FG 10 cell monolayers were incubated for 3 days with 24 h old supernatants of TMC-treated and control cells. Since Toyocamycin was always present in the medium, it was necessary to prevent its cytostatic effect on FG 10 cells. This was performed by adding adenosine (10^{-5}M) to the medium. This concentration of adenosine impeded the effect of Toyocamycin and permitted close to normal cell growth. After 3 days incubation, the media were changed and the cultures were continued until the 6th or 7th day. Foci of transformed cells were counted (Table I). It can be seen that biological infectivity of the TMC particles was 1/4th of the control, while virus production estimated by protein content of the purified virus still represented 69 % of control culture (experiment 1).

When TMC (0.2 μg/ml) was added to the control cell supernatant just before performing the biological assay, one observed a slight decrease in the number of foci on the FG 10 cells monolayer (experiment 1 + TMC). But this effect was always less pronounced than the one produced by virus grown in the continual presence of TMC (average of several experiments).

TABLE I

Measurement of the infectivity of the virus released by TMC-treated cells and control cells (see text for explanations)

	Number of foci*	Virus content**
Experiment 1		
Control virus	800	100 %
Control virus + TMC (0.2 μg/ml)	510	100 %
TMC virus	180	69 %
Experiment 2		
Control virus	210	100 %
TMC virus	2.5	39 %

* On FG 10 cell monolayers.
** Estimated by optical density at 260 nm of material banding at density 1.15-1.17 g/cc in sucrose gradients. This procedure was standardized with the method of Lowry (8), using bovine albumin and purified virus.

Experiments performed with Actinomycin D have shown that viral particles released during the early phase of the drug action are not modified in their 70S RNA content and maintain their biological activity (7). We speculated that a similar phenomenon could occur in our experiments. Therefore, cells were incubated in the presence of TMC for 4 hours. Then, the medium was replaced by fresh medium also containing the drug. Eighteen hours later, supernatants were harvested and assayed for infectivity. The results presented in the Table I (experiment 2) indicate that infectivity of TMC virions decreased by two logs, whereas the amount of virus still represented 40 % of the control.

Endogenous reverse transcriptase activity of virions released by cells cultivated in the presence of TMC for 18 hours was tested in parallel with that of control virus. Equal amounts of purified virus were added in each reaction, in order to allow a correct comparison. Results in Figure 1 indicated that TMC viral particles retained approximately 40 % of the endogenous activity of the control during a two hours reaction. This diminution can be explained either by an alteration of the enzyme or alternatively by a modification of the genomic RNA or both. When reverse transcriptase activities of TMC-treated and control viruses were compared in reactions using poly rA-oligo-dT as an exogenous template, no differences were observed, suggesting that the enzyme present in the TMC-treated virus had retained its normal activity. Another

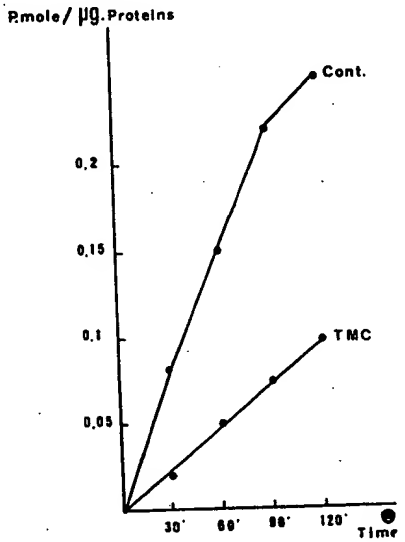

FIG. 1.

Endogenous reverse transcriptase activity of virions produced in the presence of TMC (0.2 µg/ml). Results are expressed as picomoles of [³H] TMP incorporated per µg viral proteins.

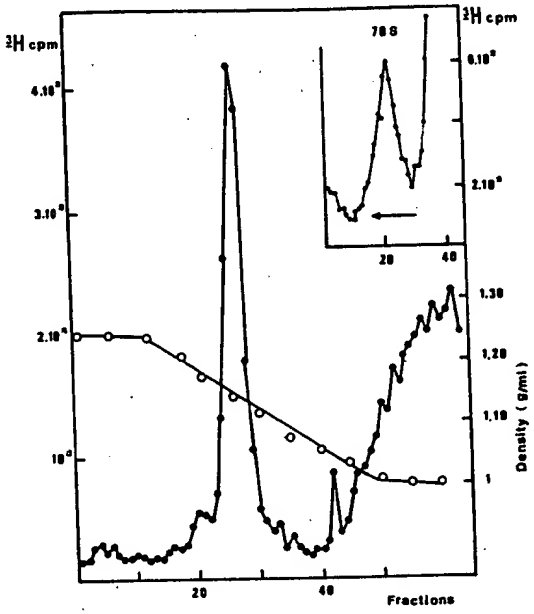

FIG. 2.

Incorporation of Toyocamycin in virus synthesized in the presence of the drug. 2.10^6 STU cells were labeled for 16 hours with 0.2 µg/ml [³H] Toyocamycin (specific activity : 10 Ci/mmole — Commissariat à l'Énergie Atomique, Saclay, France). Supernatant was treated as described in the method and the material in the peak banding at the density 1.15 g/cc was pelleted by centrifugation at 40 000 rpm (rotor 50 Titan, Beckman) for 60 mn. RNA was released from the virus pellet by 0.5 % sodium dodecyl sulfate and analyzed in a 10-30 % sucrose gradient (insert). Conditions of centrifugation were in SW-65 Beckman rotor at 44 000 rpm for 2 hours at 4 °C. The NTE buffer contained 2 µg/ml Potassium Polyvinylsulfate.

possibility could be that TMC adsorbs on viral proteins and impairs the enzymatic reaction. This was ruled out by adding 0.2 µg/ml TMC to a reaction mixture containing purified virus. No difference resulted from the presence of the drug (Data not shown).

Toyocamycin has been reported to incorporate into cellular RNA molecules (11). To detect its presence in the 70S viral RNA, cell were exposed to [³H]-Toyocamycin for 16 hours. Virus was purified and viral RNA was extracted and run in a sucrose velocity gradient (Fig. 2). The result clearly demonstrates that the radioactivity recovered in the viral particles was incorporated in the 70S viral RNA. Digestion of the RNA by 0.5N NaOH or T_2 RNAse confirmed this point (not shown).

DISCUSSION

The results presented here confirm and extend previous ones (2, 9), as we have found that TMC markedly diminished the production of viral particles in the culture media at a dose which was sufficient to inhibit the cell growth and the processing of the 45S precursor of the ribosomal RNAs. This production was never totally suppressed even by doses of TMC which destroyed the cells in less than 24 h (1 µg/ml). Accordingly, since these results mimic those obtained by Levin et al. (7) using Actinomycin D, we looked at the integrity of the virions by testing their biological capacity, reverse transcriptase activity and RNA content. The results of this investigation clearly showed that virus produced under TMC treatment was altered since it was less infectious. The endogenous activity of the reverse transcriptase was decreased, suggesting that the defect took place during the synthesis of the provirus. However, we assume that the enzyme was not impaired since most of it was translated from viral mRNAs molecules present on the polysomes before drug addition. Moreover, exogenous reverse transcriptase activity using Poly rA-oligo-dT or Poly rC-oligo-dG was entirely similar wether the enzyme consisted of control virions or Toyocamycin-treated virions. On the other hand, our data indicate that Toyocamycin was incorporated into viral RNA. The most plausible explanation is that the RNA molecules cannot be used as efficient templates for the synthesis of the viral DNA.

ACKNOWLEDGMENT

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Biomedicine, 1979, *31*, 20-23.

DE NOVO SYNTHESIS OF PURINE NUCLEOTIDES AND METABOLIC AVAILABILITY OF PHOSPHORIBOSYLPYROPHOSPHATE IN LEUKEMIC LEUKOCYTES

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SUMMARY

Among normal peripheral blood leukocytes, lymphocytes were found to contain most of the de novo purine synthesizing capacity. The rate of purine synthesis de novo was accelerated in leukocytes from patients with acute and chronic myelocytic leukemias, chronic monocytic leukemia, myelofibrosis and plasma cell leukemia, but was normal in most patients with chronic lymphocytic leukemia. The rate of de novo purine synthesis exhibited positive correlation with the percentage of immature cells in the leukocyte population. The metabolic availability of phosphoribosylpyrophosphate (PR PP) exhibited positive correlation with the rate of de novo purine synthesis. These finding suggest that the accelerated rate of de novo purine synthesis and the increased metabolic availability of PRPP are characteristic properties of he immature leukemic granulocyte.

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Reprints : O. Sperling.

RÉSUMÉ

Parmi les leucocytes normaux du sang périphérique, on a trouvé que les lymphocytes contiennent la plupart de la capacité de synthèse de novo de la purine. Ce taux de synthèse de novo de la purine est accéléré dans les leucocytes de patients atteints de leucémies myéloïdes aiguë et chronique, monocytaire chronique ou à plasmocytes, et de patients atteints de myélofibrose. Ce taux est par contre normal dans la plupart des cas de leucémies lymphoïdes chroniques.

Une corrélation positive a été établie entre le taux de synthèse de novo de la purine et le pourcentage de cellules leucocytaires immatures. La disponibilité métabolique du phosphoribosylpyrophosphate (PRPP) présente une corrélation positive avec le taux de synthèse de novo de la purine. Ces données suggèrent que le taux accéléré de la synthèse de novo de la purine et la disponibilité métabolite augmentée du PRPP sont des propriétés particulières au granulocyte leucémique immature.

INTRODUCTION

Studies in our laboratory (2) as well as elsewhere (3, 5) have established that normal peripheral blood leukocytes are capable of synthesizing de novo purine nucleotides. The present investigation was undertaken to clarify which of the cells among the normal leukocytes contain the purine synthesizing capacity and to explore the rate of activity of this pathway in leukocytes of leukemic patients. Since phosphoribosylpyrophosphate (PRPP) has been shown to be a limiting substrate for the activity of the PRPP amidotransferase, catalyzing the first-committed and rate limiting step in the pathway of purine syntheis de novo (2), the meta-

ROLOGY 98, 99–107 (1979)

The Specific Inhibition of Vesicular Stomatitis Virus Replication by Toyocamycin

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Toyocamycin, an adenosine analog, inhibited the reproduction of vesicular stomatitis virus (VSV) by greater than 99%. The drug appeared to act preferentially at the level of replication since intracellular genome length 42 S RNA synthesis and the formation of viral nucleocapsids were inhibited by 90%, whereas VSV mRNA synthesis was reduced only by 50–60%. The VSV mRNAs were all polyadenylated in toyocamycin-treated cells, although the length of their poly(A) sequences increased slightly with increasing concentrations of the drug. Toyocamycin was incorporated into all intracellular VSV RNA species. In spite of the significant substitution of toyocamycin for adenosine into both the heteropolymeric and poly(A) regions of the VSV mRNAs, they were biologically functional since the viral L, G, N, NS, and M proteins were synthesized at about 20–40% of control values, levels approximately proportional to the amount of the mRNAs present.

INTRODUCTION

Vesicular stomatitis virus (VSV), a habdovirus, is a relatively simple animal irus that contains a single stranded RNA genome of the negative sense with a molecular weight of about 4×10^6 (Wagner, 1975). Virtually all of the genetic information of he virus is transcribed into five viral nRNAs, 31, 17, 14.5, 12, and 12 S, (Moyer t *al.*, 1975; Rose and Knipe, 1975) which ode for the L, G, N, NS, and M proteins, espectively (Both *et al.*, 1975; Knipe *et al.*, 1975). There is a single initiation site for ranscription at the 3′ end of the genome esulting in the sequential appearance of the nRNAs in the order 5′ N → NS → M → G → L 3′ (Ball and White, 1976; Abraham and Banerjee, 1976). Based on this bservation, a model was proposed in which ranscription might proceed via the synhesis of a full-length (+) strand genome omplement which could subsequently be rocessed by cleavage, blocking, and nethylation to the individual functional mRNAs. However, no direct evidence yet exists that such a (+) strand full-length RNA is an obligatory intermediate in transcription.

In attempts to determine whether the processing of a precursor RNA occurs during the transcription of VSV, we have studied the effect of toyocamycin, an adenosine analog, on the reproduction of VSV. We chose this drug as a potential inhibitor of viral RNA processing since it is known to inhibit the cleavage of the 45 S ribosomal RNA precursor to its 28 and 18 S rRNA subunits (Tavitian *et al.*, 1968). Furthermore, toyocamycin also inhibits the processing of the adenovirus heteronuclear precursor RNA (McGuire *et al.*, 1972), although this is now believed to be due to the lack of addition of poly(A) sequences to the viral mRNA precursor in the presence of the drug (Swart and Hodge, 1978).

We have shown that toyocamycin completely suppressed the reproduction of VSV, although this was not due to an inhibition of RNA processing. In drug-treated cells VSV mRNA synthesis and polyadenylation were virtually unaffected,

[1] To whom reprint requests should be addressed.

while the replication of the viral genome was specifically inhibited. Although toyocamycin was incorporated in place of adenosine into all intracellular VSV RNA species, the substituted mRNAs were functional and coded for all the VSV polypeptides.

MATERIALS AND METHODS

Cells and virus. Baby hamster kidney (BHK) cells in suspension were employed for all experiments. The procedures for the growth and purification of VSV (Indiana serotype, Mudd-Summers), free of defective interfering particles, have been described previously (Banerjee *et al.*, 1974).

Preparation of labeled virus and subviral components from infected cells. BHK cells were infected with VSV at a multiplicity of infection (m.o.i.) of 10 PFU/cell as described previously (Moyer and Gatchell, 1979). At 1 hr postinfection, actinomycin D was added at a final concentration of 2 μg/ml. The actinomycin D-treated, infected cells were then incubated with toyocamycin (4 amino-5-cyano-7β-D-ribofuranosyl-pyrrolo-[2,3*d*]-pyrimidine, a gift from Dr. R. Suhadolnik, Department of Biochemistry, Temple University School of Medicine, Philadelphia, Pa.) for 30 min at either 1.0 or 2.5 hr postinfection prior to the addition of the radioactive precursors. The inhibitor was continuously present for the duration of the labeling period. The cultures were labeled with [^{3}H]uridine from either 1.5 or 3.0 hr postinfection as indicated in the text. In order to label with [^{32}P]orthophosphate (100 μCi/ml), BHK cells were starved for 24 hr prior to infection in phosphate-free minimal essential medium and then infected with virus in phosphate-free medium as described above. Labeled virus was pelleted from the supernatant fluid at 24 hr postinfection and quantitated by velocity gradient centrifugation as described by Doyle and Holland (1973). Alternatively, the infected cells were harvested, a cell extract was prepared, and viral nucleocapsids (Moyer and Gatchell, 1979) and RNAs (Moyer and Banerjee, 1975) were isolated as described previously.

Purification and analysis of viral RNA. RNA from purified virus or VSV nucleocapsids was extracted with phenol–chloroform and precipitated with ethanol. RNA annealing experiments were performed as described by Moyer and Gatchell (1979). The proportion of polyadenylated VSV 31 S and 12–18 S mRNAs was determined by oligo(dT) cellulose column chromatography as previously described (Banerjee *et al.*, 1974). Poly(A) sequences were isolated from ^{32}P-labeled VSV poly(A)$^+$ 12–18 S mRNA by digestion for 60 min with RNase A (50 μg/ml) and RNase T_1 (10 U/ml) in buffer containing 0.03 *M* sodium citrate, pH 7.2 and 0.3 *M* NaCl at 37°, and subsequently purified by oligo(dT) cellulose column chromatography. The size of the poly(A) sequences was determined by electrophoresis on 10-cm 20% polyacrylamide gels (10 g acrylamide:0.075 g bisacrylamide) containing 8 *M* urea, 10% (v/v) glycerol, and 2× electrophoresis buffer, pH 8.3. Electrophoresis was carried out at 130 V for 19 hr in buffer containing per liter: 10.8 g Tris base, 0.93 g Na_2EDTA, and 5.5 g boric acid, pH 8.3. The gels were fractionated into 1-mm samples using a Gilson gel fractionator, dissolved with 1 ml of 30% H_2O_2 at 60° for 24 hr, and counted in ASC aqueous scintillation fluid in a scintillation counter.

Base analysis of ^{32}P-labeled VSV RNAs synthesized in the presence of toyocamycin was performed after digestion of the RNA with RNase T_2 (50 U/ml), RNase A (100 μg/ml), and RNase T_1 (20 U/ml) for 16 hr at 37°. The nucleotides were then separated by high voltage electrophoresis on Whatman 3 MM paper in pyridine–acetate buffer, pH 3.5 at 3000 V for 2.5 hr.

Quantitation of VSV-specific protein synthesis. VSV infected cells either untreated or treated at 1.0 hr postinfection with 1.0 μg/ml of toyocamycin were labeled with [^{3}H]leucine (25 μCi/ml) from 1.5 to 5 hr in the presence of the drug. Cytoplasmic cell extracts were then prepared in the presence of 2 m*M* phenylmethylsulfonylfluoride. The VSV specific proteins were precipitated with antisera against VSV (kindly provided by Dr. D. F. Summers, University of Utah Medical Center) with the use of the staphylococcal protein A–antibody absorbent (Kessler, 1975). The

roteins were quantitated by electrophoresis on 7.5% polyacrylamide–SDS ube gels in phosphate buffer (Maizel, 966), or on 10% polyacrylamide–SDS lab gels according to the method of aemmli (1970).

Chemicals and enzymes. Carrier-free ^{32}P]orthophosphate and [^{3}H]leucine were urchased from New England Nuclear and 5,6-^{3}H]uridine (40 Ci/mmol) from Schwarz/ Mann. Oligo(dT) cellulose Type T_2 was purhased from Collaborative Research and ctinomycin D from Sigma Chemical Company. Enzymes were purchased as described previously (Moyer and Banerjee, 1976).

RESULTS

The Effect of Toyocamycin on VSV Progeny Production and RNA Synthesis

To study the effect of the adenosine analog, toyocamycin, on the reproduction of VSV, several concentrations of the drug were first tested on both uninfected and VSV infected cells. As had been shown for L cells (Tavitian *et al.*, 1968), total RNA synthesis in uninfected sBHK cells was inhibited 50–60% within 4 hr compared to the control by 0.1 and 1.0 μg/ml of toyocamycin (Fig. 1A). Increasing the drug concentration to 10 μg/ml inhibited RNA synthesis by 90% with no further inhibition at higher concentrations. In contrast, total RNA synthesis in VSV-infected cells was virtually unaffected by 0.1 μg/ml toyocamycin when it was added at either 1 or 3 hr postinfection (Figs. 1B and C). Addition of 1 or 10 μg/ml toyocamycin caused an inhibition of the total synthesis of VSV-specific RNA of about 40–60% relative to the control at both times of the infectious cycle.

The effect of toyocamycin specifically on the reproduction of VSV was subsequently determined. VSV-infected cells were treated at 1 hr postinfection with varying concentrations of the drug and labeled with [^{3}H]uridine. The level of virus released into the supernatant was quantitated at 24 hr. While 0.1 μg/ml of toyocamycin resulted in a moderate inhibition of VSV production, higher concentrations of the drug completely (>99%) abolished virus synthesis (Table 1).

In VSV-infected cells there are three major classes of virus specific RNAs that can be detected: 42 S genome RNA and the 31 and 12–18 S classes of mRNAs. We next determined the effect of toyocamycin on the synthesis of each class of VSV RNA by analysis on SDS–sucrose gradients of cytoplasmic extracts derived from infected cells that had been treated with different concentrations of the drug at either 1 or 2.5 hr postinfection. The data from these experiments are summarized in Table 2. While 0.1 μg/ml of toyocamycin had little effect on the levels of any of the VSV RNAs, 1.0 and 10 μg/ml had a drastic inhibitory effect, particularly on the synthesis of the 42 S RNA. These results suggest that the primary effect of the drug appears to be an inhibition of the synthesis of intracellular 42 S RNA (90%) together with a concomitant inhibition of virus production. The synthesis of VSV mRNA, while reduced, remained at a level slightly higher than that observed for the primary transcription of VSV obtained in the presence of an inhibitor of protein synthesis (Marcus *et al.*, 1971).

The Synthesis of VSV Nucleocapsids in Toyocamycin-Treated Cells

Since a small amount of genome length VSV RNA was detected in toyocamycin-treated VSV-infected cells, we wanted to determine if this RNA was incorporated normally into subviral particles. Infected cells were treated with toyocamycin at 1 hr postinfection and at 5.0 hr cytoplasmic extracts were prepared. Subviral components were separated on velocity gradients and the data presented in Fig. 2 indicate that, compared to the control, most of the residual 42 S VSV RNA (~10%, Table 2) synthesized in drug-treated cells was found in the 120 S viral nucleocapsids (~10%). The VSV 42 S RNA from the drug-treated, infected cells was isolated, denatured by dimethylsulfoxide, and further purified by gradient centrifugation. Annealing of this material to unlabeled (−) strand 42 S genome RNA from purified virus showed

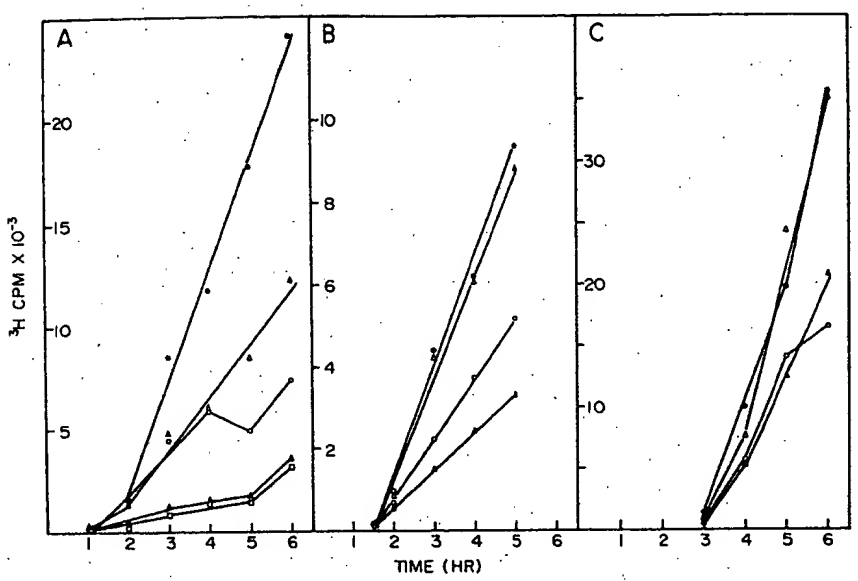

FIG. 1. Total RNA synthesis in toyocamycin-treated uninfected BHK cells or cells infected with VSV. Uninfected BHK cells were treated with toyocamycin at 0.5 hr (A) and VSV-infected cells were treated with the drug at 1.0 hr (B) or 2.5 hr (C) postinfection. The cultures were labeled 30 min later with [^{3}H]uridine (5 μCi/ml). At the indicated times, duplicate 100-μl samples were removed and TCA-precipitable counts were determined. The total counts in toyocamycin-treated cells at 0 (●), 0.1 (△), 1.0 (○), 10.0 (▲), and 50.0 (□) μg/ml.

that the intracellular 42 S RNA contained about 17% (+) strand RNA with the remainder of the RNA of the (−) strand sense (Table 3). Thus, a limited amount of *de novo* synthesis of 42 S RNA of both strand senses occurred in toyocamycin-treated cells.

The Effect of Toyocamycin on the Polyadenylation of the VSV mRNAs

Although the overall size of the VSV mRNA species synthesized in toyocamycin-treated cells appeared unaffected (Table 2), we then determined whether the polyadenylation pattern of the mRNA was altered. Swart and Hodge (1978) have previously reported that adenovirus-specific mRNA was not polyadenylated when infected cells were treated with toyocamycin. We found, however, that the polyadenylation of the VSV mRNAs, as measured by their binding to an oligo(dT) cellulose column, was unaffected by high concentrations (1–10 μg/ml) of toyocamycin (Table 4). Furthermore, analysis of the poly(A)$^+$ RNA species from drug-treated cells by velocity gradient sedimentation showed that all the individual species (31, 17, 14.5, and 12 S) identical in size to the control VSV mRNAs were present (data not shown).

TABLE 1

THE EFFECT OF TOYOCAMYCIN ON VSV PROGENY PRODUCTION

Toyocamycin (μg/ml)	Progeny virus	
	cpm	Percentage
No drug	23,338	100
0.1	13,439	57.6
1.0	189	0.8
10.0	221	0.95

Although the amount of polyadenylation of the mRNAs remained unchanged, the treatment of infected cells with toyocamycin caused an increase in the size of the poly(A) sequence. The poly(A)$^+$ ^{32}P-labeled VSV 12–18 S mRNA was purified from cells treated with 0, 1, or 10 μg/ml of toyocamycin at 2.5 hr postinfection as described under Materials and Methods. The RNA was digested with RNases A and T_1 to remove the heteropolymeric region and the remaining poly(A) sequences were purified by oligo(dT) cellulose column

hromatography. The size of the poly(A) ras determined by electrophoresis on 20% olyacrylamide–urea gels (Fig. 3). The oly(A) sequences of mRNA from the con-rol culture were heterogeneous with a iaximum length of about 200 nucleotides as as been previously reported (Villarreal nd Holland, 1973; Ehrenfeld, 1974; Moyer *t al.*, 1976). The poly(A) sequences of nRNA from toyocamycin-treated infected ells, however, were larger and more iomogeneous in size. At 10 and 1 μg/ml of oyocamycin the average poly(A) size was 40 and 260 nucleotides, respectively. The ncrease in size correlated with the amount f toyocamycin incorporated into the RNA see below) at this time of infection.

ncorporation of Toyocamycin into VSV-Specific RNAs

Since toyocamycin inhibited primarily VSV genome RNA synthesis rather than

TABLE 2

THE EFFECT OF TOYOCAMYCIN ON INTRACELLULAR VSV-SPECIFIC RNA

Toyocamycin (μg/ml)	RNA species[a]		
	42 S (%)	31 S (%)	12–18 S (%)
(A) Drug added 1.0–5.0 hr			
No drug	100	100	100
0.1	79.2	93.2	87.5
1.0	15.4	40.1	50.1
10.0	10.9	19.7	35.6
(B) Drug added 2.5–5.5 hr			
No drug	100	100	100
0.1	66.4	96	118
1.0	10.2	18.1	27.9
10.0	14.5	29.3	41.2

[a] VSV-infected cells were treated with different concentrations of toyocamycin at the times indicated and labeled with [^{3}H]uridine (5 μCi/ml). Cytoplasmic cell extracts were prepared, dissociated with SDS, and the RNA species were separated by gradient centrifugation on parallel 15–30% (w/v) SDS–sucrose gradients at 22,500 rpm for 17 hr at 24° in the SW41 rotor. The total RNA (cpm) of each size class was determined and expressed as a percentage of the RNA in untreated infected cells.

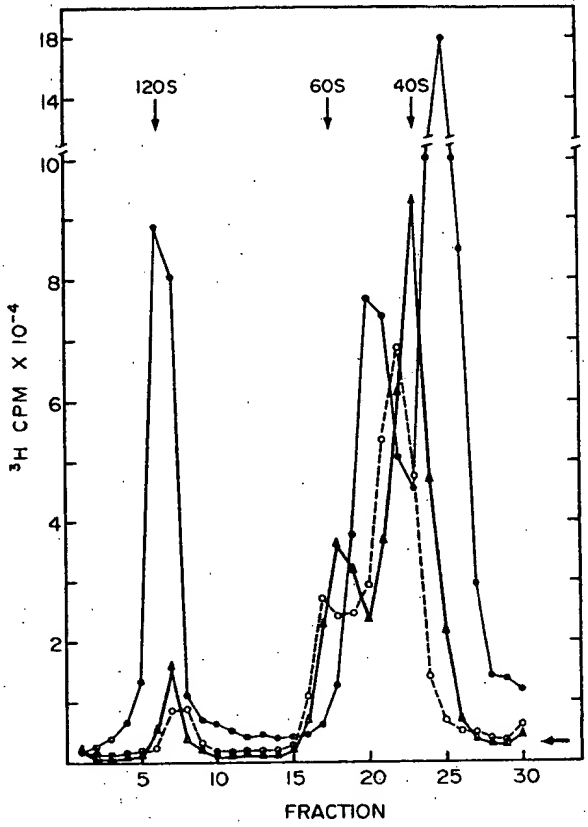

FIG. 2. The effect of toyocamycin on VSV nucleocapsid synthesis. BHK cells infected with VSV were treated with toyocamycin at 0 (●), 1.0 (○), and 10.0 (▲) μg/ml at 1 hr postinfection and labeled with [^{3}H]uridine (10 μCi/ml) from 1.5 to 5.0 hr. Cytoplasmic cell extracts were prepared and analyzed on parallel 15–30% (w/v) sucrose NEBS gradients at 19,000 rpm for 17 hr at 4° in the SW41 rotor as described previously (Moyer and Gatchell, 1979). Each gradient fraction was tested for TCA-precipitable counts. Sedimentation is from right to left.

mRNA synthesis, we wanted to determine if the drug was differentially incorporated into the heteropolymeric regions of any of these RNA species. Infected cells were treated with various concentrations of toyocamycin at either 1 or 2.5 hr postinfection and labeled with ^{32}P. The VSV 42, 31, and 12–18 S RNA species were isolated and purified as described under Materials and Methods. Base analysis was performed for each RNA following digestion with RNases T_2, T_1, and A. Figure 4A shows a typical pattern obtained following electrophoresis of the 12–18 S mRNA isolated from cells treated at 1 hr with toyocamycin at 1.0 μg/ml. In addition to the four major nucleotide 3′-monophosphates, there was

TABLE 3

DETERMINATION OF THE STRAND SENSE OF VSV NUCLEOCAPSID RNA SYNTHESIZED IN THE PRESENCE OF TOYOCAMYCIN

Samples[a]	42 S RNA[b] cpm	42 S RNA[b] Percentage
Not annealed	4887	100
+ RNase	11	0.22
Annealed 24 hr	4604	100
+ Unlabeled 42 S genome RNA (μg) + RNase		
0.15	817	17.7
0.30	818	17.8
0.60	819	17.8
+ Unlabeled VSV mRNA (μg) + RNase		
0.15	3374	73.3
0.30	3453	75.0
0.60	3572	77.6

[a] Annealing reactions were performed as described by Moyer and Gatchell (1979).

[b] [^{3}H]Uridine-labeled intracellular 42 S RNA from cells treated with toyocamycin (10 μg/ml) was isolated by SDS gradient centrifugation, denatured, and purified by SDS–sucrose gradient centrifugation.

the appearance of an additional significant peak migrating between pA and pG with the mobility reported for toyocamycin 3′-monophosphate (Swart and Hodge, 1978). Quantitation of this peak suggests a 26% substitution of adenosine by toyocamycin. To substantitate this identification, the toyocamycin derivative (indicated by the brackets, Fig. 4A) was eluted and treated with alkali. This treatment was previously shown to slow the electrophoretic mobility of this derivative (Swart and Hodge, 1978). Following electrophoresis, the alkali-treated VSV derivative now migrated close to the origin (Fig. 4B) as described above.

The amount of toyocamycin incorporated into each of the VSV RNA species was determined in a similar manner. When the drug was added at 1 hr postinfection, there was an increase in the amount of toyocamycin incorporated into each class of RNA with an increase in the concentration of the drug (data not shown) and this was also roughly proportional to the amount of inhibition of viral mRNA synthesis (Table 2). However, when toyocamycin was added later in infection, we observed more incorporation of the drug into RNA and a larger inhibition of mRNA synthesis at 1.0 μg/ml (47% of the adenosine substituted) than at a concentration of 10 μg/ml (32% of the adenosine substituted). The reason for the disparity at different times of infection is not apparent. Toyocamycin was not incorporated preferentially into any part of the messenger RNAs since both the total mRNAs and their poly(A) sequences contained an equal proportion of toyocamycin substituted for adenosine residues (data not shown).

VSV Protein Synthesis in Toyocamycin-Treated Infected Cells

VSV mRNAs synthesized in the presence of toyocamycin were found to have incorporated significant quantities (25 to 47%) of the drug in place of adenosine residues. Since inhibitors of protein synthesis have been shown to specifically inhibit VSV replication (Marcus *et al.*, 1971), we wanted to determine if the inhibition of replication we observed in drug-treated cells was due to the production of nonfunctional

TABLE 4

THE EFFECT OF TOYOCAMYCIN ON THE POLYADENYLATION OF VSV RNA BY OLIGO(dT) CELLULOSE CHROMATOGRAPHY[a]

Sample	Not bound (cpm)	Bound cpm	Bound Percentage
VSV 12–18 S mRNA			
Control	8,800	23,160	72.5
+ Toyocamycin			
1 μg/ml	6,946	24,372	78
10 μg/ml	22,780	146,760	86
VSV 31 S mRNA			
Control	3,368	7,268	68.3
+ Toyocamycin			
1 μg/ml	3,268	8,064	72.2
10 μg/ml[a]	17,484	28,874	62.2

[a] The RNA examined in this experiment was from a preparation labeled under different conditions than the other samples.

ıRNA. Therefore, we studied virus-pecific protein synthesis in this system. 'he total incorporation of [^{3}H]leucine in oyocamycin-treated (1 μg/ml) infected ells was 40% of that in control infected ells from 1.0 to 5 hr postinfection. A cyto-)lasmic cell extract was prepared as de-;cribed under Materials and Methods and he VSV-specific proteins were precipitated vith antisera against whole virus. The pro-;eins were separated and quantitated by ;lectrophoresis on SDS–polyacrylamide ;els (Table 5). All five VSV proteins were found in drug-treated cells. The N, NS, and L proteins were synthesized at about 40% of the control level, while both the M and G proteins were reduced to 20% of the control. The preparation of VSV antisera that was utilized in these experiments apparently inefficiently precipitated the L protein, since the amount of L protein in the cell extract of toyocamycin-treated cells was 36% compared to the control, while in the antibody-precipitated sample it was low and variable in both the control and drug-treated samples. In contrast, similar quantitation for the other viral proteins was obtained from both the cell extract and the antibody-precipitated samples.

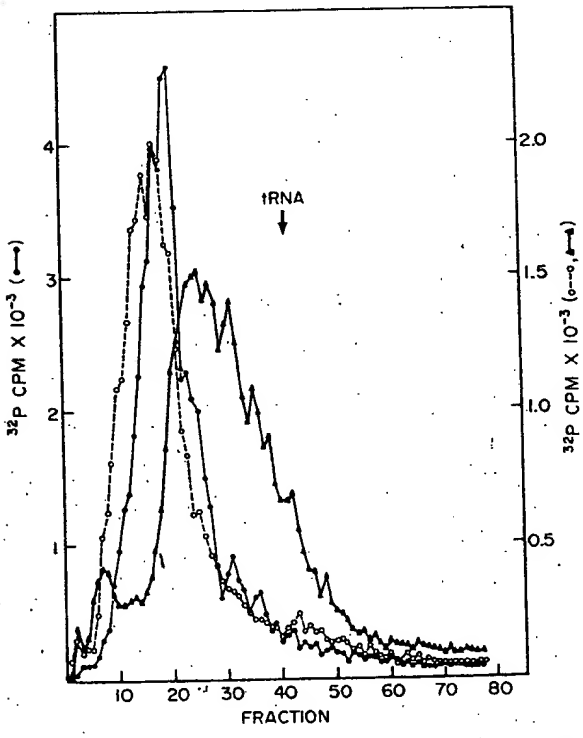

FIG. 3. The size of the poly(A) sequences in VSV mRNA isolated from toyocamycin-treated cells. ^{32}P-Labeled poly(A) sequences from VSV 12–18 S mRNA were isolated and purified as described. The samples were analyzed on parallel 20% polyacrylamide–8 *M* urea gels as described under Materials and Methods, but are plotted for convenience on one graph. Poly(A) sequences from cultures treated with toyocamycin at 0 (▲), 1.0 (○), and 10.0 (●) μg/ml. Electrophoresis is from left to right.

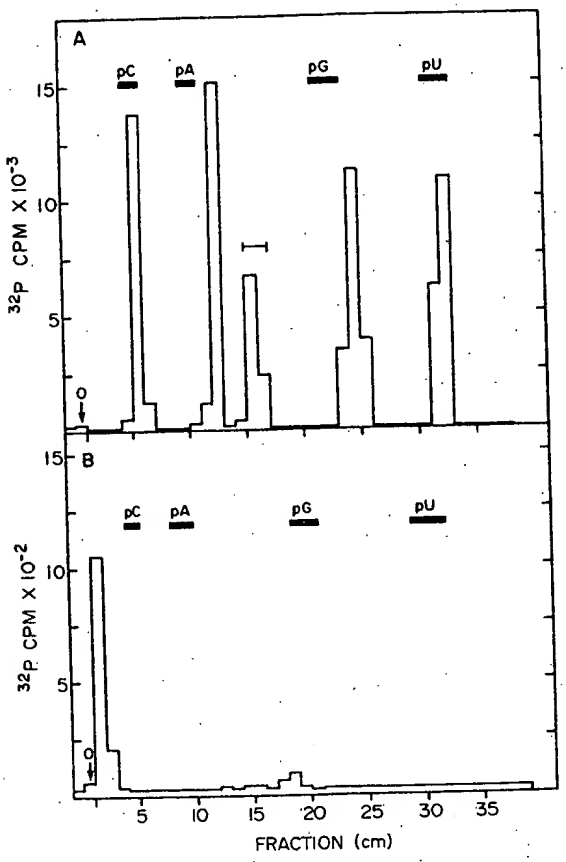

FIG. 4. The base analysis of VSV RNA species by high voltage electrophoresis. Poly(A)-containing, ^{32}P-labeled VSV 12–18 S mRNA was isolated and purified as described under Materials and Methods. The RNA was digested with RNases T_2, T_1, and A and the products were analyzed by electrophoresis on Whatman 3MM paper (A). The material indicated by the brackets (fractions 16 and 17) was eluted and treated with 0.3 *M* KOH for 16 hr at 37°, neutralized, and subsequently analyzed by electrophoresis (B).

DISCUSSION

The addition of the adenosine analog, toyocamycin, to VSV-infected cells was found to inhibit virus production by greater than 99% (Table 1). The drug appeared to preferentially inhibit viral replication by re-

TABLE 5

VSV PROTEIN SYNTHESIS IN TOYOCAMYCIN-TREATED INFECTED CELLS

Sample	Anti-VSV antibody-precipitated protein (cpm)[a]				
	L	G	N	NS	M
Control	950	9,207	28,219	3,349	2,836
+ Toyocamycin (1 μg/ml)	351	2,360	11,573	1,496	617

[a] The [^{3}H]leucine-labeled antibody-precipitated proteins, except for L protein, were analyzed by SDS–polyacrylamide tube gel electrophoresis. The analysis of the L protein in a cell extract was performed on a SDS–polyacrylamide slab gel. The L protein band was located by fluorography and the band excised and counted in scintillation fluid.

ducing progeny genome RNA synthesis by 90% (Table 2). The level of VSV mRNA synthesis observed was lowered but was that approximately expected for transcription from the unamplified template (primary transcription). A more detailed examination of the VSV mRNA showed that all the expected species were represented and that they contained poly(A) sequences (Table 4) with a mean length somewhat higher than what is normally observed (Fig. 3). The effect of toyocamycin in one other virus system has been examined but quite different effects were noted. In the case of adenovirus, toyocamycin blocked virus growth by preventing the polyadenylation of adenovirus heteronuclear RNA (Swart and Hodge, 1978). Likewise, cordycepin, the adenosine analog 3′-deoxyadenosine, also had different effects on polyadenylation in these two virus systems. Cordycepin blocks adenovirus adenylation (Philipson *et al.*, 1971) while the drug has virtually no effect on the polyadenylation or growth of VSV (Ehrenfeld, 1974). The fact that VSV mRNA polyadenylation is insensitive to two different drugs, both of which inhibit adenovirus adenylation, suggests that the poly(A) sequences are added by different mechanisms or enzymes in these two systems.

Toyocamycin has also been shown to inhibit the processing of the mammalian ribosomal precursor RNA, presumably by its misincorporation into the RNA in place of adenosine (Tavitian *et al.*, 1968). One reason these studies were initiated was to probe the possibility that the VSV mRNAs are also derived from a larger RNA precursor. All VSV mRNA transcription arises from a single initiation site and each of the five messages shares a common adenosine-rich 5′-terminal pentanucleotide, pApApCpApG (Rhodes and Banerjee, 1976; Rose, 1978) which might serve as a recognition site for processing. Our hope was that the incorporation of toyocamycin into the viral RNA might prevent any hypothetical processing. Toyocamycin, although readily incorporated, had no effect on the normal formation of the VSV mRNAs. Therefore, our results suggest either that RNA processing, if present in the VSV system, is much different than that for the ribosomal RNAs, or that processing does not occur in the formation of the VSV mRNAs which would favor a mechanism where the individual mRNA species arise by a "stop–start" process (Ball and White, 1976; Abraham and Banerjee, 1976).

Other possibilities to explain the *in vivo* action of toyocamycin to inhibit VSV genome RNA replication could be, first, a direct effect of the drug on genome RNA synthesis, or second, that some aspect of mRNA translation is affected because the RNA contains large quantities of misincorporated toyocamycin. The latter possibility is unlikely for although toyocamycin was extensively incorporated into the VSV mRNAs (Fig. 4), the substituted messengers were translated in infected cells to yield all the VSV proteins (Table 5) in amounts roughly proportional to the levels of their mRNAs. Furthermore, normal functionality of at least some of the viral proteins was indicated by the fact that the low amount of genome RNA synthesized in drug-treated cells was properly assembled into nucleocapsids containing newly formed VSV proteins. Analysis of the strand sense of the residual VSV 42 S RNA that forms in toyocamycin-treated cells showed about 20% (+) and 80% (−) strands (Table 3); a ratio similar to that found for the intracellular 42 S RNA of

productively infected cells (Soria *et al.*, 1974). Therefore, the block in VSV replication by toyocamycin appears to occur by preventing the amplification of progeny RNA synthesis which indicates the existence of a more complex replication process than has been previously postulated.

ACKNOWLEDGMENTS

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